Original Article

Real-time Quantitative Polymerase Chain Reaction Assay for Detecting 1p and 19q Codeletion in Glioma

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Abstract

Background: Glioma is a type of tumor that occurs in the brain and spinal cord. Gliomas begin in the gluey supportive cells (glial cells) that surround nerve cells and help them function. Gliomas are classified according to the type of glial cell involved in the tumor, as well as the tumor's genetic features, which can help predict how the tumor will behave over time and the treatments most likely to work. Among the molecular markers for the classification of glioma, loss of the 1p/19q fragments is by far the most well-characterized and most widely studied. In this study, we used real-time polymerase chain reaction (PCR) as an alternative technique to fluorescence *in situ* hybridization (FISH) to detect 1p/19q deletion mutations in adult gliomas. **Methods:** This was a cross-sectional study. Specific primers were designed for target genes represented for 1p and 19q areas. Real-time PCR was performed for 60 formalin-fixed paraffin-embedded samples which were randomly divided into two groups: whole tissue DNA extraction and tumor-only area DNA extraction. FISH was used as a confirmation method. **Results:** Real-time PCR results from DNA isolated from whole tissue showed a low similarity with FISH results (56.6% for 1p and 66.6% for 19q), while real-time PCR results from DNA of tumor-only area showed high similarity with FISH results for both markers (80%). For samples with 1p/19q deletion, real-time PCR showed a relatively low sensitivity as this technique only detected 5 out of 11 samples with 1p/19q deletion. **Conclusions:** Using DNA extracted from the tumor-only area, real-time PCR has a similarity of 80% compared with FISH in detecting 1p/19q deletion.

Keywords: 1p/19q deletion, gliomas, real-time polymerase chain reaction

Introduction

Central nervous system (CNS) malignancies are among the cancers with the worst prognosis, and the highest mean years of potential life lost.[1,2] Gliomas are the most common primary CNS tumors,[3] with an estimated annual incidence of 6.6 per 100,000 persons in the United States.[4] About half of all newly diagnosed gliomas are glioblastoma, the most aggressive type of brain cancer with an average patient survival time of 14–17 months in the clinical trials[5] and approximately 12 months in population studies.[6] The World Health Organization's latest classification system of gliomas in 2016 includes Grade II and Grade III astrocytomas, oligodendrogliomas, glioblastomas, and gliomas glial cells in children.[7,8]

Histological classification has for decades been considered the "gold standard" for the diagnosis of gliomas, but reclassification is significantly dependent on the observer, especially in diffuse gliomas.[9] Studies have revealed that the molecular classification of gliomas correlates better with clinical outcomes than the histological classification.[10,11]

Some prominent molecular markers for gliomas include *IDH1* mutations in diffuse gliomas,[2] fusion mutations of the *BRAF* gene in astrocytomas,[12] *MGMT* gene methylation in glioblastoma,[13] and codeletion of chromosomes 1p/19q in oligodendroglioma.[14] Among the molecular markers mentioned above, loss of the 1p/19q fragments is by far the most well-characterized and most widely studied.[14,15]

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Quick Response Code:

Website: https://journals.lww.com/BBRJ

DOI: 10.4103/bbrj.bbrj_196_23

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How to cite this article: Vo NV, Truong DD, Xinh PT, Vu HA. Real-time quantitative polymerase chain reaction assay for detecting 1p and 19q codeletion in Glioma. Biomed Biotechnol Res J 2023;7:563-8.

Submitted: 10-Jul-2023; **Revised:** 07-Sep-2023;
Accepted: 13-Sep-2023; **Published:** 23-Dec-2023.

This deletion is caused by a full-arm balanced translocation of chromosomes 1 and 19, resulting in the formation of two derivative chromosomes. One of these derivative chromosomes including 1p and 19q (der[1,19] [p10;q10]) is usually lost.[16] Codeletion of 1p/19q is a genetic marker of oligodendroglioma with a prevalence of over 80% among pure oligodendrogliomas and approximately 40% among oligoastrocytomas.[17-20] Almost all tumors with deletion of 1p/19q are accompanied by point mutations on 2 genes *IDH1/IDH2*.[21] Interestingly, among tumors with the 1p/19q codeletion, tumors with the polyploid phenotype generally have a relatively worse prognosis than those without polyploidy.[22] The most commonly applied techniques to detect 1p/19q loss are loss of heterozygosity (LOH)[23] and fluorescence *in situ* hybridization (FISH).[24] LOH requires control of blood DNA from the same patient, but blood samples are not routinely stored in the clinical setting. FISH 1p/19q codeletion detection is commonly used in clinical laboratories but is expensive and requires highly experienced personnel to ensure data standardization and accuracy. Real-time polymerase chain reaction (PCR) can be considered an accurate technique that allows the detection of this 1p/19q codeletion, independent of LOH or FISH and using only tumor sample DNA. Real-time PCR is based on selected marker genes and reference genes, by quantitative analysis of the absolute ratio of marker and reference gene copy numbers in the DNA sample (normal ratio 1/1, loss <0.8, and repeat >1.2).[25] Real-time PCR is dependent on gene copy number (reference gene vs. marker gene), theoretically applicable to all loci.

Methods

Ethical consideration

The study approved by the Ethical Committee of the university of Medicine and Pharmacr at Ho Chi Minh City , approval number 248/HDDD-DHYD,April 15, 2021.

Type of sampling and reasons for selection

Paraffin-embedded tissue samples obtained from surgical resection of Glioma were sent to Center for Molecular Biomedicine–University of Medicine and Pharmacy at Ho Chi Minh City, for molecular testing. All paraffin block samples were obtained from patients diagnosed with brain tumors. We included 60 samples which were divided into two groups: whole tissue extraction and tumor-only area extraction.

Informed consent

We retrospectively used paraffin-block embedded tissues from consented patients.

Inclusion criteria

We collected paraffin-embedded samples from patients diagnosed with brain tumors and the tissue specimen must be large enough (1 cm^2), with the percentage of tumor cells from at least 30% of the specimen.

Exclusion criteria

Samples containing a lot of necrotic tissue or tumor cell percentage less than 30% were not selected for further analyses.

Primer design and evaluation

Primers were designed using CLC Main Workbench V.5.5 software (QIAGEN, Hilden, Germany) with normal parameters such as 18–24 bp long, melting temperatures (Tm) between 59°C and 68°C and higher than the Tm of any secondary structures in the template, GC density between 50% and 60%, no repeated sequence, product size between 80 and 150 bp. Sanger sequencing was used to clarify the specificity of primers.

Fluorescence *in situ* hybridization

Tumor areas need to be selected under the light microscope and then performed FISH with Vysis 1p36/1q25 and 19q13/19p13 FISH Probe Kit (Abbott, USA). FISH analysis is performed using a dual-color approach for chromosomes 1 and 19 separately. Target probes hybridize to subtelomeric 1p36 and 19q13 in combination with control probes on 1q and 19p, respectively.

DNA extraction

DNA isolation was performed according to the protocol supplied with ReliaPrep™ Formalin-Fixed Paraffin-Embedded gDNA Miniprep System (Promega, USA). The amount of purified DNA was quantified on a spectrophotometer (NanoDrops 2000c Spectrophotometer, Thermo Scientific, Wilmington, DE, USA). An absorbance ratio of 260–280 nm (A260/A280) was used to assess the quality of isolated DNA. Samples having an A260/A280 ratio of 1.8–2.0 were deemed to be of good quality.

Real-time polymerase chain reaction

Real-time PCR primer mix was used for selected marker genes in 1p (E2F2 and NOTCH2), 19q (PLAUR), and reference genes (ERC2 and SPOCK1). Real-time PCR was performed with real-time PCR instrument (Eppendorf, USA). PCR was carried out in a 20 µl reaction volume composed of 1 µl of sample DNA (30 ng/µl), 10 µl of TB Green Master Mix (Takara Bio, Japan), 1.5 µl of the primer mix, and 7.5 µl of water. The sequences of the original primers are shown in Table 1.

Thermal cycling conditions included an initial denature temperature of 98°C in 3 min, followed by 45 cycles of denature temperature of 98°C in 15 s, annealing temperature of 60°C in 20 s, and elongation temperature of 72°C in 40 s then final elongation temperature of 72°C in 2 min. A melting curve cycle was included for each run to ensure no formation of primer dimer after 45 cycles. All genes were quantified at least twice in separate runs, to show assay reproduction with 20% standard deviation, then the mean copy number of each gene was taken for analysis, by calculating the ratio of each marker to the reference gene. A normal ratio is considered 1.0 and any ratio <0.80 is considered deletion of the region of interest.[25]

Table 1: Primer sequences for real-time polymerase chain reaction

Gene name	Primer sequence 5'–3'	
	Forward	Reverse
E2F2	GAAGTCTACCTGTGCCCAGA	GATGCTGTGGGCTCCATGAT
NOTCH2	TACAGGAGAGGACTGCCAGT	GTAAACCCGACTTGACAGGT
PLAUR	AAGGAGAAGAGCTGGAGCTG	TTGCCCTGGTTGCACAAGTC
ERC2	TCACCAATCTGGAAGGTAGC	CATAGACAGAGTCTTGCCTG
SPOCK1	ACAGACAAGGAGTTGCGGAA	TTACTGCCTTGGGCTGTGTT

E2F2: E2F transcription factor 2, NOTCH2: Notch receptor 2, PLAUR: Plasminogen activator, urokinase receptor, ERC2: ELKS/RAB6-interacting/CAST family member 2, SPOCK1: SPARC (osteonectin), cwcv and kazal like domains proteoglycan 1

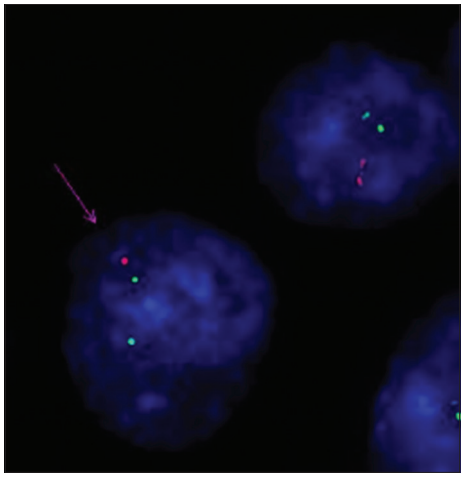

Figure 1: Fluorescence *in situ* hybridization result shows abnormal cell carrying 1p36 deletion (1R2G) on the left with the arrow, normal cell (2R2G) on the right (×100). R, red; G, green

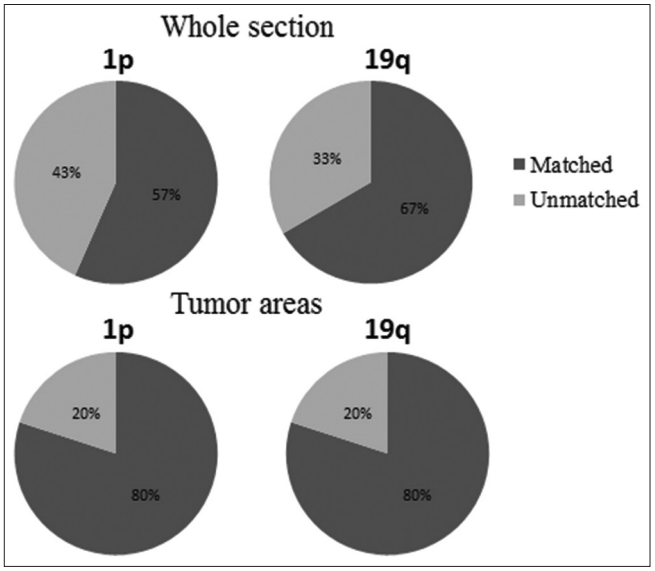

Figure 2: Correlation of fluorescence *in situ* hybridization and real-time polymerase chain reaction results for 1p/19q deletion using the whole tissue and the tumor-only area

Results

Fluorescence *in situ* hybridization detection of 1p and 19q loss

In interphase nuclei of normal cells hybridized with the 1p36 and 1q25 probes, 2 red and 2 green signals (2R2G) will be observed indicative of 2 intact copies of chromosome 1. In an abnormal cell with a deletion in the 1p36 region, fewer than 2 red signals will be observed [Figure 1]. Similar result analysis also applies to 19q13 and 19p13 probes. For evaluation, the signal ratio is assessed around 100–200 adjacent, nonoverlapping interphase nuclei, and the results are expressed as percentage. If more than 30% of "deleted" nuclei found, the sample is considered to show a deletion of 1p36 and 19q13.[14]

FISH result showed 41.6% (25/60) of 1p deletion, 48.3% (29/60) of 19q deletion, and 38.3% (23/60) sample had 1p/19q codeletion.

Real-time polymerase chain reaction showed a low sensitivity with whole tissue DNA extraction

A ratio of 1:1 between selected genes and reference genes in autosomal chromosomes is expected in normal cells while changes in this ratio would suggest either deletion or amplification. The average of two ratios of ERC2 and NOTCH2 was used to identify 1p status for all tumors. With consideration of 10%–20% variation inherited with real-time PCR, the mean values of the marker and reference ratio were taken for determination of deletion (<0.8) or amplification (≥1.2).[25] As shown in Table 2, real-time PCR results of 1p and 19q markers showed 56.6% (17/30) and 66.6% (20/30) compatible with FISH, respectively, suggesting a low sensitivity for this method. We hypothesize that the percentage of tumor cell with 1p/19q deletion and the normal cell maybe the main cause for this low sensitivity, and to overcome this issue we selected tumor tumor-only area under the light microscope and used that tumor-only area for DNA extraction before performing real-time PCR.

Tumor-only area DNA extraction showed higher concordance between real-time polymerase chain reaction and fluorescence *in situ* hybridization

By doing tumor area selection, real-time PCR results of both 1p and 19q showed 80% (24/30) compatible with that of FISH and also proved that the percentage of tumor cells (with 1p/19q deletion) and normal cells really affect the sensitivity of PCR [Figure 2 and Table 3].

Table 2: Real-time polymerase chain reaction result, mean ratios of 1p/19q marker genes to each reference genes for whole tissue extraction

Number	Sample	qPCR 1p36	FISH 1p36	qPCR 19q13	FISH 19q13
1	AST 71	1.63	Del	1.4	Del
2	AST 72	0.94	Del	0.81	Del
3	AST 74	1.23	Nml	0.76	Del
4	AST 78	0.56	Del	0.73	Del
5	AST 82	0.86	Del	0.82	Nml
6	AST 96	1.23	Nml	0.99	Nml
7	AST 97	1.64	Del	0.8	Del
8	AST 101	1.16	Del	0.84	Del
9	AST 104	0.97	Nml	0.67	Nml
10	AST 106	1.14	Nml	1	Nml
11	AST 108	0.92	Nml	0.78	Nml
12	AST 109	0.92	Nml	1.09	Nml
13	AST 110	1.68	Nml	1.18	Nml
14	AST 112	0.8	Del	0.77	Del
15	AST 113	0.88	Del	0.55	Del
16	AST 117	1.13	Del	0.94	Del
17	AST 116	1.35	Nml	1.11	Nml
18	AST 118	0.26	Del	0.12	Del
19	AST 119	1.03	Del	0.92	Del
20	AST 121	0.74	Nml	0.57	Del
21	AST 132	1.29	Nml	0.59	Del
22	AST 135	1.97	Nml	1.5	Nml
23	AST 136	1.28	Nml	0.76	Del
24	AST 139	1.03	Nml	0.7	Nml
25	AST 183	0.65	Nml	0.57	Del
26	AST 186	2.29	Nml	1.94	Nml
27	AST 222	1.19	Del	0.59	Del
28	AST 223	0.94	Del	1.04	Del
29	AST 225	0.78	Del	0.61	Del
30	AST 229	1.72	Nml	0.86	Nml

Del: Deletion, Nml: Normal, FISH: Fluorescence *in situ* hybridization, qPCR: Quantitative polymerase chain reaction

Table 3: Real-time polymerase chain reaction result, mean ratios of 1p/19 marker genes to each reference genes for tumor-only area extraction

Number	Sample	qPCR 1p36	FISH 1p36	qPCR 19q13	FISH 19q13
1	AST 73	0.75	Del	0.59	Del
2	AST 85	0.85	Nml	0.86	Nml
3	AST 100	1.86	Nml	0.98	Nml
4	AST 146	1.3	Nml	1.19	Nml
5	AST 147	0.95	Del	0.85	Del
6	AST 148	0.8	Nml	0.93	Nml
7	AST 149	1.59	Nml	1.21	Nml
8	AST 151	0.94	Nml	0.93	Nml
9	AST 152	1.84	Nml	1.52	Nml
10	AST 154	0.75	Del	1.34	Nml
11	AST 158	0.93	Nml	1.07	Nml
12	AST 159	2.41	Nml	1.77	Nml
13	AST 241	0.75	Del	0.59	Del
14	AST 244	1.13	Nml	1.57	Nml
15	AST 253	1.45	Nml	0.98	Nml
16	AST 266	0.93	Del	1.16	Del
17	AST 270	1.47	Nml	1.17	Nml
18	AST 273	1.19	Nml	1.25	Nml
19	AST 274	1.06	Del	1.27	Del
20	AST 275	0.79	Del	0.75	Del
21	AST 276	1.03	Nml	0.84	Nml
22	AST 277	1	Del	0.88	Del
23	AST 278	1.43	Nml	0.84	Nml
24	AST 280	0.97	Nml	0.51	Del
25	AST 282	0.75	Del	0.61	Del
26	AST 284	1.38	Nml	1.16	Nml
27	AST 292	1.34	Nml	0.92	Nml
28	AST 294	1.26	Nml	1	Nml
29	AST 296	1.77	Del	1.54	Del
30	AST 299	1.61	Del	1.4	Del

Del: Deletion, Nml: Normal, FISH: Fluorescence *in situ* hybridization, qPCR: Quantitative polymerase chain reaction

Discussion

The codeletion of 1p/19q is considered a strong prognostic factor in gliomas, especially for oligodendrogliomas, being associated with a longer progression-free and overall survival of the patients. Patients with 1p/19q codeleted tumors show increased overall survival and are more likely to respond to chemotherapy.[26] In addition to its value as a prognostic and predictive marker, 1p/19q codeletion also plays a role in the diagnosis of morphologically uncharacteristic cases which mimic oligodendroglioma.[27]

FISH is an approved method for investigation of 1p/19q status in paraffin-embedded tissues.[28] Our FISH result of 60 gliomas samples shows 41.6% of 1p deletion, 48.3% of 19q deletion, and 38.3% of 1p/19q codeletion. This result is similar to other studies,[17,18] suggesting that our FISH procedure is reliable and can be used as a standard for real-time PCR results.

Real-time PCR has grown into a potent investigative and diagnostic tool with the capacity to generate accurate and repeatable results. The goal of this method is to monitor the amplification products produced during each cycle of the PCR reaction by combining the chemistry of the polymerase chain reaction with the usage of fluorescent reporter molecules. The procedure is also quick, affordable, reproducible, low risk of contamination, and requires less hands-on time. Most notably, real-time PCR analysis can begin with very little amounts of DNA.[29]

In the first phase of the study, the whole tissue was selected for real-time PCR, with low similarity to the FISH results. Therefore, in the later phase of the study, we selected tumor-only area for subsequent real-time PCR reactions.

Our real-time PCR data show that whole tissue extraction, especially samples with low percentage of 1p/19q deletion tumor cells, could affect the sensitivity of PCR. In detail, real-time PCR analysis based on the ratio of 1p/19q fragments of deletion cells and normal cells, tumor specimens with low number of 1p/19q deletion cells could show a false negative.

This limitation is the major problem for this technique if we want to develop it to become a clinical test. However, one modification that we can do to minimize the false negative was tumor-only area selection, by this way, we could dramatically eliminate normal cells and increase the accuracy of this method.

Although tumor-only area selection might enrich the number of tumor cells and decrease the false negative result, we still observe a low sensitivity for samples with 1p/19q loss. For samples without 1p/19q loss, the PCR result was quite compatible with that of FISH; in contrast, real-time PCR detected only 5 out of 11 cases with 1p/19q codeletions which were confirmed by FISH among 30 cases tested.

The result showed that this technique has high specificity but limited sensitivity; although the similarity between PCR and FISH is 80%, the deviation of 20% needs to be noticed when real-time PCR is applied clinically.

Conclusions

Tumor-only area selection can improve the result of real-time PCR and bring FISH matching rates up to 80% for detection of 1p/19q codeletion in glioma and should be considered for clinical application.

Out come of the study

The Real-time PCR demonstrated to be sensitive and rapid methods to detect mutation deletion in studied gliomas sample.

Rationale of the study

In this study, we used real-time PCR as an alternative technique to FISH to detect 1p/19q deletion in adult gliomas.

Limitation of study

The sensitivity of real-time PCR was only improved when using DNA isolated from the tumor-only area. This requires a pathologist, who is not always available in a molecular biology laboratory, to identify the area with tumor cells.

Acknowledgments

We would like to thank Dr. Tran Kim Tuyen of Cho Ray Hospital for assisting the research team in sample collection.

Financial support and sponsorship

We would like to thank the University of Medicine and Pharmacy at Ho Chi Minh City for the financial support of this research.

Conflicts of interest

There are no conflicts of interest.

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